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TECHNICAL REPORT

Discovery and Validation of Proteomic Biomarkers for Radiation Exposure

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February 2012

HDTRA1-07-1-0030

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14. ABSTRACT
The overall objective of this project is to further advance the fundamental knowledge base for new and existing proteomic biomarkers that can assess the type and amount of radiation exposure rapidly (in about one hour), sensitively (at protein concentration of ~0.1 ng/mL or less), and inexpensively.

15. SUBJECT TERMS

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I. Objective

The overall objective of this project is to further advance the fundamental knowledge base for new and existing proteomic biomarkers that can assess the type and amount of radiation exposure rapidly (in about one hour), sensitively (at protein concentrations of ~0.1 ng/mL or less), and inexpensively. In particular, we aim at discovering and validating proteomic biodosimetry biomarkers by combining *ex vivo* protein chips for human serum sample protein profiling and *in vivo* analysis of mouse model under controlled radiation exposures. If fully validated, the proposed technology and biodosimetry markers will likely lead to significant military, commercial, and clinical applications, and help safeguard our national security against potential accidents and terrorist attacks, including those involving radiological weapons.

The objectives of this project include the following:

1. Demonstrate microwell protein chips which can readily immobilize protein capture probes relevant to biodosimetry.
2. Demonstrate that the analytical sensitivity of the protein chip to be ~0.1 ng/mL.
3. Discovery and validation of biomarkers of radiation exposure in human peripheral blood mononuclear cells (PBMCs).
4. Develop a point of care diagnostic device which can be adapted for radiation exposure triage.

II. Status of effort

Exposure to ionizing radiation (IR) generally produces few immediate visible clinical signs. Nevertheless, depending on the dose, IR can severely damage vital physiological functions within hours to days after exposure and produce long-lasting health consequences among survivors. Thus, in the event of a nuclear or dirty bomb attack, a major challenge in the future is to identify civilian populations or military personnel who might have been exposed to radiation but have not yet manifested signs and symptoms. For example, protein CDKN1A inhibits the activity of cyclin-CDK2 or -CDK4 complexes, and functions as a regulator of cell cycle progression. The CDKN1A protein levels in an exposed patient can change markedly as a function of radiation dosage, thus providing an effective dosimetry for radiation triage after a radiological incident. We have shown that CDKN1A expression level indeed varies monotonically with radiation dose from 0 to 4 Gy in ex vivo radiated human blood samples (**Figure 1**).

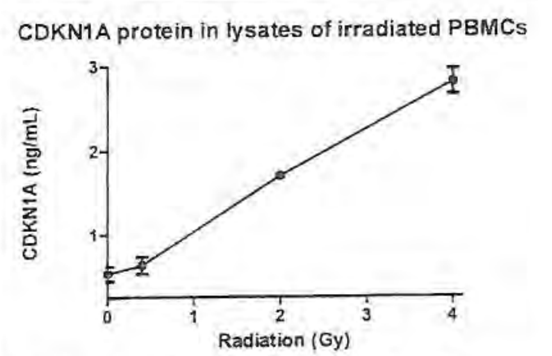


Figure 1: CDKN1A protein levels as a function radiation dosage, as measured by K. Wilson at Stanford using ELISA kits.

We have developed magneto-nano chips suitable for assaying human cell lysate from human blood samples after radiation exposure *ex vivo* or mouse serum samples from mouse blood sample after whole body radiation *in vivo*. The chips are functionalized with capture agents for well known radiation biomarkers (e.g., cyclin-dependent kinase inhibitor 1A (CDKN1A), or C-reactive protein (CRP)), to construct an early, sensitive, and accurate measure

of radiation dosage from human peripheral blood samples post radiation. The calibration curve for human CRP detection is shown in **Figure 2**.

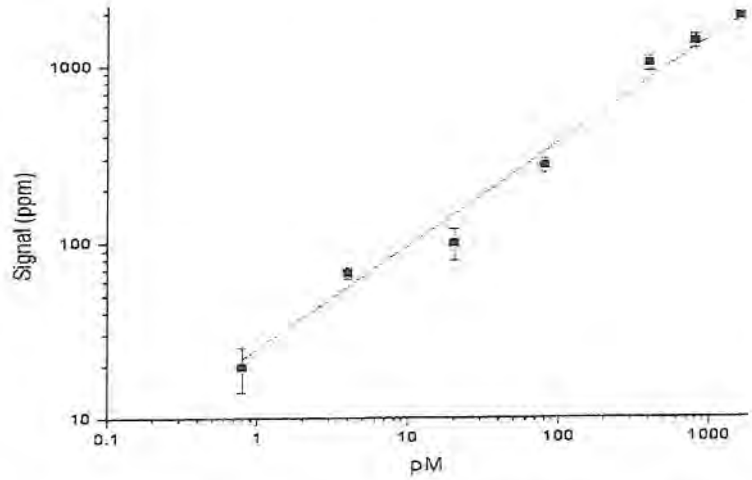


Figure 2: Stanford curve for human CRP detection on magnetic protein chips. The limit of detection (LOD) is about 0.8 pM, which is appreciably better than those of Life diagnostics CRP test (Blakely et al.): LOD ~ 0.8 ng/mL = 6 pM, and of Roche Diagnostics CRP (Latex) (k083444) : LOD ~ 0.2 ug/mL = 1.5 nM.

III. Accomplishments

Our magnetic nanotag-based technology uses giant magnetoresistance (GMR) spin valve sensor array to quantitate the number of magnetic nanoparticle labels selectively bound to their surfaces. Analogous to sandwich ELISA, the chip is spotted with capture antibody, incubated with protein standard or other analytes and then, with biotinylated detection antibody. The sandwich complex is tagged by streptavidin-conjugated MACS nanoparticles. Results were compared to a commercially-available ELISA kit.

We were able to reliably quantitate CDKN1A levels down to concentrations of ~100pg/mL with magnetic nanotag-based chips, comparable to or slightly better than ELISA (**Figure 3**). The same technology has been used to detect other biomarkers (e.g., CEA) down to 1 pg/mL level (**Figure 4**). Furthermore, the magnetic chip achieved a superior dynamic range of quantifiable protein concentrations in approximately one-third the assay time as ELISA. This paper appeared in Nature Medicine, 2009.

In order to facilitate effective deployment of magnetic chip technology in the field by non-technical users, it is important that the assays be integrated into an ultraportable and battery-powered test module. This advance removes the need for a constant supply of electricity or a designated laboratory. Since, the form factor of GMR sensors is on the nanoscale, by miniaturizing the electronic components of the current setup and utilizing nanosensors, we are able to replace an entire laboratory full of equipment with a handheld and battery powered device (**Figure 5**). No lasers or expensive charge coupled device (CCD) cameras are required for the platform, which uniquely positions GMR based biosensors for ultraportable, POC applications. This work will be presented at ISCAS Conference in Paris in May 30-June 2, 2010.

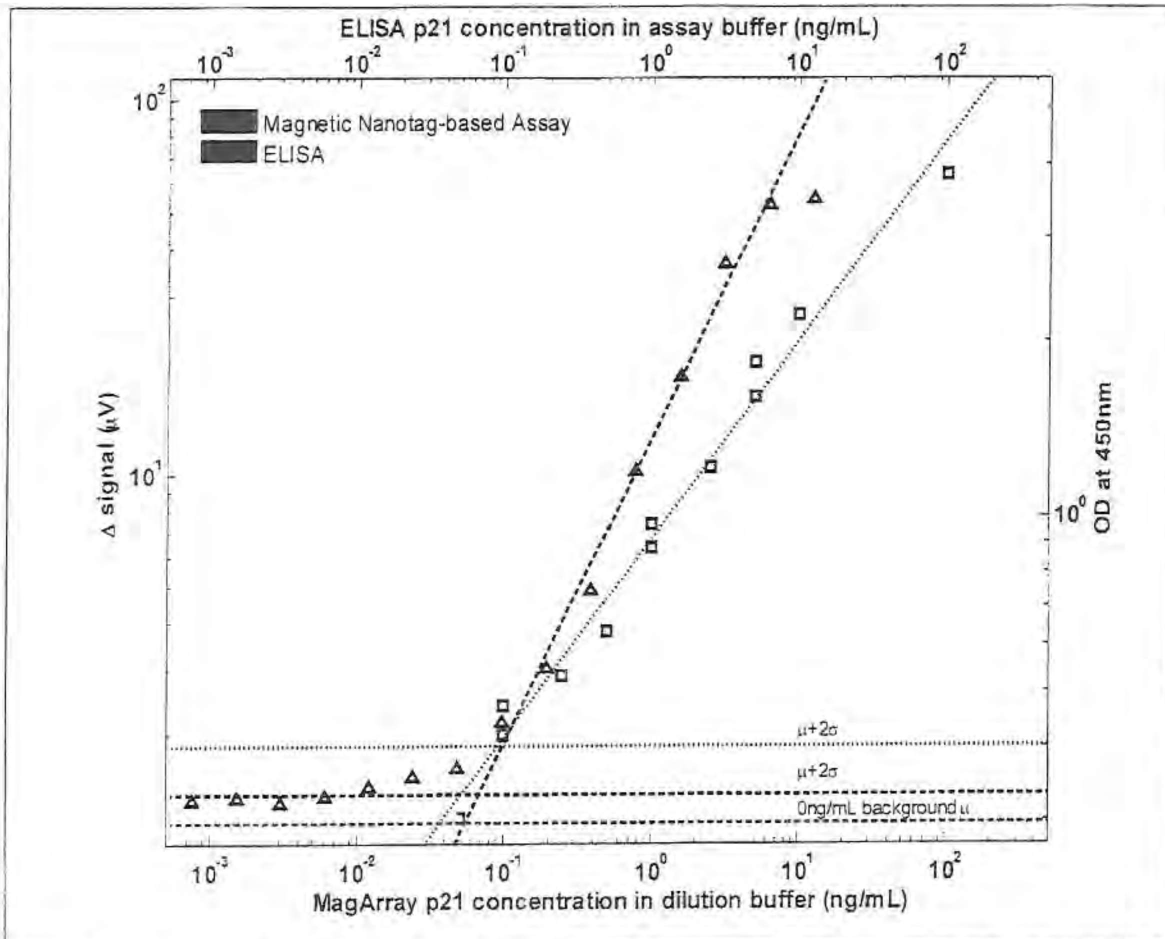


Figure 3: Sensitivity and dynamic range of CDKN1A radiation marker in magneto-nano sensor and ELISA, respectively.

Magnetic nanotag-based protein chips are capable of quantitating radiation biomarkers at sensitivities comparable to or better than ELISA over a larger concentration range and in considerably less time. This technology has the promise to enable fast, sensitive biodosimetry in the event of radiation exposure. Our work under DTRA grant laid the foundation for Stanford University to successfully bid for a major BARDA contract for developing POC and high throughput systems through the Rapid and Accurate Proteomic Index Dosimetry (RAPID) Consortium (News release attached).

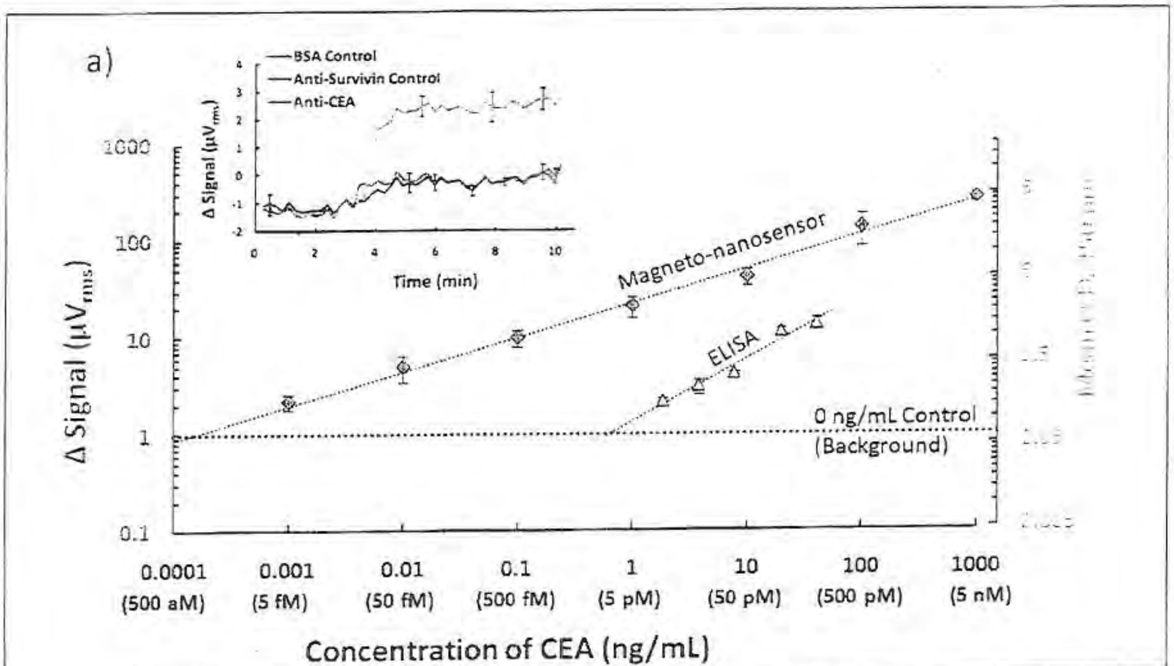


Figure 4: Sensitivity and linear dynamic range (on a log-log plot) of CEA marker in magneto-nano sensors and ELISA. Superimposed serial dilution curves of CEA detection on the magnetic nanosensor (blue) and ELISA (green) comparing the linear dynamic range and the lower limit of detection in 0.1% BSA in PBS (the same antibody pairs were used for both assays). μVrms is the unit of GMR sensor signal presented on the left in blue, while O.D. (optical density) at 450 nm wavelength is the unit of ELISA readout presented on the right in green. By superimposing the background signal (red) for each technology, an accurate comparison can be made. Here, the background is defined as the average signal with no
(0 ng/mL) CEA spiked into the reaction well for each technology plus 2 standard deviations. The error bars represent +/- 1 SD. Insert: Real-time monitoring of change in voltage over time when 5 fM CEA (blue) is spiked into the reaction well when compared to the BSA control (red) and a non-complementary Anti-Survivin antibody control (grey). The error bars represent +/- 1 SD.

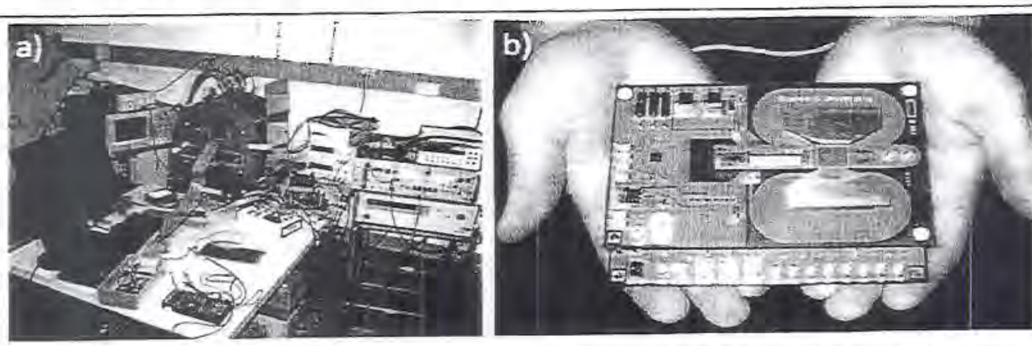


Figure 5 | Demonstration of miniaturization from initial laboratory test station (**a**) into the novel handheld device (**b**). All components of the laboratory setup have been miniaturized and incorporated into an ultraportable platform.

IV. Personnel Supported

Primary personnel:
Shan Wang, PhD (co-PI)
Joseph Wu, MD PhD (co-PI)
Drew Hall (graduate student, Wang lab)
Mei Huang, PhD (post-doc, Wu lab)

Personnel partially supported by the grant:
Kitch Wilson, MD (graduate student, Wu lab)
Dokyoon Kim (graduate student, Wang lab)
Lisa Chen (graduate student, Wang lab)
Shu-Jen Han (graduate student, Wang lab)

V. Publications

1. S. X. Wang and G. Li, Advances in GMR Biosensors with Magnetic Nanoparticle Tags: Review and Outlook, Invited Review for *Advances in Magnetics* of *IEEE Trans. Magn.*, vol. 44, no.7, 1687-1702, 2008.
2. S. X. Wang, "Giant Magnetoresistive Biochips for Genetic and Protein Assays," *Digest of the 66th Device Research Conference*, Santa Barbara, CA, June 23-5, 2008.
3. S. X. Wang, "Giant magnetoresistive biochips for biomarker detection and genotyping: an overview (invited)," AIP Conference Proceedings, *European Symposium on Biomagnetism and Magnetic Biosystems Based on Molecular Recognition Processes (ESF-EMBO Symposium)*, Sant Feliu de Guixols, Spain, Sept 22-27, 2007.
4. S. J. Osterfeld and S. X. Wang. MagArray Biochips for Protein and DNA Detection with Magnetic Nanotags: Design, Experiment, and Signal to Noise Ratio, book chapter to appear in *Microarrays: New Development Towards Recognition of Nucleic Acid and Protein Signatures*, edited by Kilian Dill, Robin Liu, and Piotr Grodzinski, Springer Verlag/Kluwer, 2007.
5. S-J Han, Ph.D. Thesis, CMOS INTEGRATED BIOSENSOR ARRAY BASED ON SPIN VALVE DEVICES, Summer'07.
6. Lisa Y. Chen, Kitchener D. Wilson, Sebastian J. Osterfeld, Heng Yu, Joseph C. Wu, Shan X. Wang. A sensitive magnetic nanotag-based protein chip for high-throughput biodosimetry of the ionizing radiation biomarker CDKN1A (p21). Late Health Effects of Ionizing Radiation: Bridging the Experimental and Epidemiologic Divide (Georgetown University, Washington DC, May 2009).
7. S. J. Osterfeld, H. Yu, R. S. Gaster, S. Caramuta. L. Xu, S.-J. Han, D. A. Hall, R. J. Wilson, S. Sun, R. L. White, R. W. Davis, N. Pourmand, and S. X. Wang, "Multiplex Protein Assays Based on Real-Time Magnetic Nanotag Sensing," *PNAS*, 105, 20637-20640 (4 pp, published online Dec.12), 2008.
8. Drew A. Hall, Richard S. Gaster, and Shan X. Wang, GMR Biosensors, book chapter in *Spin Transport and Magnetism in Electronic Systems*, Editors: Evgeny Tsymbal and Igor Žutić, Taylor & Francis, in press, 2010.
9. D. A. Hall, R. S. Gaster, S. X. Wang, B. Murmann, "Portable biomarker detection with magnetic nanotags," Proceedings of International Symposium on Circuits and Systems

(ISCAS), Paris, France, May 30-June 2, 2010, in press.

10. K. D. Wilson, N. Sun, M. Huang, W. Y. Zhang, A. J. Lee, Z. Li, S. X. Wang, J. C. Wu, "Effects of ionizing radiation on self renewal and pluripotency of human embryonic stem cells," *Cancer Research*, in press.
11. D. A. Hall, R. S. Gaster, S. J. Osterfeld, B. Murmann, and S. X. Wang, "GMR biosensor arrays: correction techniques for reproducibility and enhanced sensitivity," *Biosensors and Bioelectronics*, 25, 2177-2181, 2010.
12. D. A. Hall, R. S. Gaster, T. Lin, S. J. Osterfeld, S. Han, B. Murmann, and S. X. Wang, "GMR biosensor arrays: a system perspective," *Biosensors and Bioelectronics*, 25, 2051-2057, 2010.
13. Richard S. Gaster, Drew A. Hall, Carsten H. Nielsen, Sebastian J. Osterfeld, Heng Yu, Kathleen E. Mach, Robert J. Wilson, Boris Murmann, Joseph C. Liao, Sanjiv S. Gambhir, and Shan X. Wang, "Matrix-insensitive protein assays: pushing the limits of biosensors in medicine," *Nature Medicine*, 15, 1327-1332, 2009.

VI. Interactions/Transitions

Shan Wang gave the following invited talks in major meetings:

1. S. X. Wang, "Magneto-Nano Protein Chip and Multiplex Sorter for Monitoring Tumor Markers," *NCI Alliance for Nanotechnology in Cancer Annual Meeting*, Manhattan Beach, California, Oct.19-22, 2009. (Also moderated a Working Group session on In-Vitro Diagnostics of Cancer.)
2. S. X. Wang, "Functional Synthetic Antiferromagnetic Nanoparticles for Magnetic Manipulation and Biomolecule Detection" ECI Conference: Nanotechnology for the Study of Cellular and Molecular Interactions, Il Ciocco Hotel and Conference Center, Barga (Tuscany), Italy, June 14-June 18, 2009.
3. S. X. Wang, "Protein Assays Based on Magnetic Nanotags and GMR Sensors: A New Tool for Fighting Cancer and Rapid Triaging", Symposium on Bionanomagnetism, *International Conference on Magnetics (Intermag)*, Sacramento, CA, May 4-8, 2009.
4. S. X. Wang, "A new tool for cancer early detection and therapy: ultrasensitive and multiplex protein assay based on magnetic nanotechnology", *Canary Foundation Annual Conference*, Stanford, CA, May 4-5, 2009.
5. S. X. Wang, "NanoMagnetic Biochips — A New Tool for Fighting Cancer and Rapid Triaging", *37th Annual Northern California Electronic Materials Symposium*, Santa Clara, Apr. 10, 2009.
6. S. X. Wang, "A new tool for cancer detection and therapy monitoring: ultrasensitive and multiplex protein assay based on magnetic nanotechnology", The inaugural *Skippy Frank Translational Medicine Fund Multidisciplinary Cancer Conference*, The Quadrus Center, Palo Alto, CA, January 16th and 17th, 2009.
7. S. X. Wang, "Magnetic Nanoparticles for Biomolecular Detection, Manipulation, and Imaging," Asian Magnetics Conference, Bushan, Korea, Dec. 10-13, 2008.
8. S. X. Wang, "Magneto-nano DNA and protein chips for multiplex molecular diagnostics," *MRS Fall Meeting*, in a special Symposium FF: Nanofunctional Materials, Structures and Devices for Biomedical Applications, Boston, Dec. 1-5, 2008.
9. S. X. Wang, "Rapid and multiplex molecular diagnosis and therapeutic monitoring of cancer based on real-time magnetic nanotag sensing," *National Cancer Institute (NCI)*

Translational Science Meeting, Washington, DC, November 7-9, 2008.

10. S. X. Wang, "Molecular diagnostics and nanomagnetic biosensors," Invited Tutorial, *American Physical Society March Meeting*, New Orleans, March 9-14, 2008.
11. S. X. Wang, "CMOS integrated magnetic biochip for cancer diagnostics," FA-04 in an invited symposium, *The International Conference on Magnetism and Magnetic Materials (MMM)*, Tampa, Nov. 5-9, 2007.
12. S. X. Wang, "Magnetic Nanotechnology for Cancer Diagnostics and Therapy Monitoring," *NCI Alliance for Nanotechnology in Cancer Annual Meeting*, Chapel Hill, North Carolina, Oct.16-18, 2007. (Also moderated a panel discussion on Nanotechnology Devices for Early Diagnosis of Cancer.)
13. S. X. Wang, "CMOS integrated magnetic biochip with high density spin valve sensor arrays," *European Symposium on Biomagnetism and Magnetic Biosystems Based on Molecular Recognition Processes (ESF-EMBO Symposium)*, Sant Feliu de Guixols, Spain, Sept 22-27, 2007.
14. S. X. Wang, "Nanomagnetic biosensors and biochips for pathogen detection and cancer diagnostics," Defense Science Research Council (DSRC) meeting, Santa Cruz, California, July 19, 2007.
15. S. X. Wang, "Nanomagnetic biosensors: status and outlook," *The 6th International Storage Technology Symposium (ISTS)*, Kalamata, Greece, June 17 - 22, 2007.
16. S. X. Wang, "Design, fabrication, and application of nanomagnetic biosensors and biochips," *International Symposium on Advanced Magnetic Materials and Applications (ISAMMA)*, Jeju, Republic of Korea, May 28 – June 1, 2007.
17. S. X. Wang, "Magnetic nanotechnology for early diagnosis and therapy of cancer," BIO International Convention, Boston, MA, May 6-9, 2007.
18. S. X. Wang, "Spin valve sensors for ultrasensitive detection of superparamagnetic nanoparticles for biological applications", *MRS Spring Meeting*, San Francisco, April 9-13, 2007.

NanoLab (Lab on a stick) invention by Gaster and Hall, graduate students from Wang Group, won BMEidea Competition (1st prize) and IEEE Change the World Competition (1st prize). It was widely reported in public media, including San Jose Mercury News (June 24, 2009) and Stanford Report (July 23, 2009).

Magnetic multiplex protein assay from Wang Group (PNAS, Dec. 30, 2008) was widely reported in public media, including Stanford Report (Dec. 1, 2008), World Journal (Dec. 4, 2008), ABC-KGO TV (Dec. 18, 2008), MIT Technology Review (Dec. 19, 2008), and Genome Technology (Jan. 28, 2009).

VII. New Discoveries, Inventions, or Patent Disclosures

1. D.A. Hall, R.J. Gaster, S.J. Osterfeld, S.X. Wang, "Temperature and drift compensation in GMR devices," US Provisional Patent Application 61/209,829, filed Mar. 10, 2009.
2. R.J. Gaster, D.A. Hall, S.X. Wang, "WASH-FREE ANALYTE DETECTION ASSAY," US Provisional Patent Application 61/168,922, filed April 13, 2009.
3. R.J. Gaster, D.A. Hall, S.X. Wang, "Methods and devices for detecting the presence of an analyte in a sample," US Patent Application 12/759,584; filed April 13, 2010. (Wash free patent)

4. R.J. Gaster, D.A. Hall, S.X. Wang, "Methods and devices for detecting the presence of an analyte in a sample," PCT Application ***(pending); filed April 13, 2010. (Wash free patent)
5. D.A. Hall, R.J. Gaster, S.J. Osterfeld, S.X. Wang, "Temperature and drift compensation in GMR devices," US Patent Application ***(pending); filed Mar. 10, 2010.
6. D.A. Hall, R.J. Gaster, S.J. Osterfeld, S.X. Wang, "Temperature and drift compensation in GMR devices," PCT Application US2010/000742; filed Mar. 10, 2010.

VIII. Honors/Awards

• Kitch Wilson receives 2007-2010 Bio-X graduate student fellowship.

• Shan Wang receives 2007-2008 Obducat Award (1st Prize).

• Shan Wang is elected Fellow of the Institute of Electrical and Electronics Engineers (IEEE), 2009.

• Joseph Wu has received 2008-2013 NIH Director's New Innovator Award - National Institutes of Health

• Richard Gaster and Drew Hall won BMEidea Competition 1st Prize Award (Shan Wang as mentor), 2009.

• Drew Hall and Richard Gaster won IEEE President's Change the World Competition 1st Prize Award (Shan Wang as advisor), 2009.

• Stanford University and Lawrence Berkeley National Lab are awarded a BARDA contract on radiation triage, 2009. (See attached news release.)

• Shan Wang receives Gates Foundation Grand Challenge Explorations Award, 2010.

X. News Release on BARDA Contract

Enclosed as a separate file.

XI. Quad Chart

Enclosed as a separate file.

Discovery and Validation of Proteomic Biomarkers for Radiation Exposure (Grant Number HDTRA1-07-1-0030)

Profs. Shan Wang and Joe Wu, Stanford University

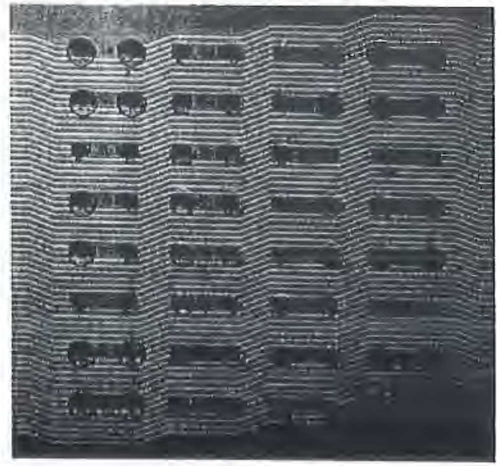
Feb. 1, 2007 to Jan. 31, 2010

Description of Effort: We aim at discovering and validating proteomic biodosimetry biomarkers by combining ex vivo protein chips for serum sample protein profiling and in vivo analysis of mouse model under controlled radiation exposures.

Challenges:

- Sensitivity/specificity of magneto-nano protein chips
- Identification and validation of specific biomarkers

64-Sensor Magneto-Nano Chip



Status of effort: Pilot study on CDKN1A radiation marker and CEA marker show a sensitivity of 0.1 ng/mL and 1 pg/mL. CRP radiation marker evaluated. NanoLab (Lab on a stick) invention won IEEE Change the World Competition (1st prize), reported in San Jose Mercury News. Won major BARDA Contract.

Personnel Supported: 2 professors, 1 Post-Doc, 4 Graduate Students supported fully or partially.

Publications & Meetings: 6 patent applications, 18 publications, 5 meetings including ECI Conf., Intermag, Canary Foundation, etc.

Major Goals/Milestones

- 1/15/08. Magneto-nano chips at 0.1 ng/mL detection.
- 7/15/08. Candidate biodosimetry proteomic biomarker panel identified.
- 11/15/09. Refining and validating the magneto-nano-chip-based protein assay w/ human serum samples.

Funding Profile

Y1: $166,897; Y2: $165,910; Y3: $165,910

PI: Shan Wang, sxwang@stanford.edu, 650 723 8671

Stanford Report, January 26, 2010

Stanford-led research team aims for rapid detection of radiation dose

Researchers think blood proteins may hold key to developing instruments for use by first-responders, labs in the event of nuclear incidents.

BY DAVID ORENSTEIN

To develop a fast, cheap and accurate technology for determining the level of radiation exposure victims might suffer in a nuclear incident, Stanford is leading a new federally funded consortium of academic, government and industry researchers. The contract provides $4.6 million in its first year, and up to $38 million over five years if the first year's results are sufficiently promising.

"The current 'gold standard' for assessing radiation doses requires several days of processing time," said Shan Wang, a Stanford professor of materials science and engineering and of electrical engineering, who leads the consortium. "We propose to develop two devices – one for high-throughput screening in the lab and one for handheld use at the point of incident – with an assay time of 20 to 30 minutes and at low cost."

Stanford is among the nine institutions around the country to have earned research contracts of various sizes from the Biomedical Advanced Research and Development Authority (BARDA), as it seeks to grapple with the prospect of radiation exposure. BARDA, which is part of the U.S. Department of Health and Human Services, announced the contracts on Dec. 18, 2009. Wang said the work of the consortium began almost simultaneously.

The devices the consortium hopes to produce would be able to sort through blood samples of victims on the scene swiftly, as well as in the lab, looking for concentrations of particular proteins, or "biomarkers," that reliably reflect the dose of radiation a person has received. The idea is reflected in the consortium's name: RAPID, for "Rapid and Accurate Proteomic Index Dosimetry."

The research question for the first year of RAPID is whether the radiation biomarkers provide accurate dose information. If so, the next four years would involve creating and validating prototype devices that could be used by first responders and eventually approved by the Food and Drug Administration.

Wang and his team have a track record in this area. In recent research Wang's group has made major advances in building systems that use magnetically sensitive chips and specially treated, molecule-sized magnetic "nanoparticles" to detect very low levels of protein biomarkers for cancer. He also has been working with medicine and radiology Assistant Professor Joe Wu on a radiation biomarker validation project funded by the Defense Threat Reduction Agency.

Wu will continue to work with Wang in the new consortium. Joining them as co-principal investigator is Andrew Wyrobek, a radiation biologist at Lawrence Berkeley National Laboratory.

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